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Epipharynx shape as a tool to reveal differentiation patterns between insect sister species: insights from *Onthophagus taurus* and *O. illyricus* (Insecta: Coleoptera: Scarabaeidae)

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Abstract

We evaluated the potential of the adult epipharynx to reveal interspecific differentiation patterns in closely related *Onthophagus* species. Although easy to analyse, this structure has received little attention in Scarabaeidae taxonomy, probably because a qualitative inspection of its shape often does not yield differences between congeneric species. The polyphenic sister species *Onthophagus taurus* Schreber, 1759 and *O. illyricus* Scopoli, 1763 were chosen as a case study. They are extremely similar, to the extent that in some cases they cannot be identified unambiguously without the help of biomolecular analysis. In this study, a combination of linear measurements and a landmark-based approach was employed to quantify inter- and intraspecific shape variation in the epipharynx of specimens sampled at the same study site. Our results showed that the epipharynx is a monomorphic structure: its shape does not vary as a function of sex or male phenotype. In males, epipharynx shape does not change with head shape or horn length. The close proximity of the epipharynx to the horns and the synchronous developmental patterns of these two structures suggest that a developmental trade-off may act between them. Despite these predisposing conditions, however, our results suggest that epipharynx size is not subject to costs associated with horn development, and that the trait is highly canalised. Surprisingly, when using geometric morphometrics the epipharynx appears to be a better tool than genitalia for discriminating between the two sister species.

Keywords: Sister species; Horn polyphenism; Sexual dimorphism; Geometric morphometrics; Static allometry; Trade-off

Introduction

Among insects, the dung beetle species *Onthophagus taurus* Schreber, 1759 and *O. illyricus* Scopoli, 1763 are considered the epitomes of male polyphenism, and are emerging as models for evo-devo studies aimed at clarifying the mechanisms underlying the rise of evolutionary novelties (Moczek 2008a, 2008b). In both species large, 'major' males bear horns, whereas smaller, 'minor' males are hornless or exhibit strongly reduced horns (Paulian 1935; Paulian and Baraud 1982; Cook 1987). This ecophenotypic variation arises because male adult body size is determined primarily by larval feeding conditions; only males with a body size exceeding a critical threshold value develop large horns (Emlen 1994; Hunt and Simmons 1997, 1998, 2000, 2002; Moczek 1998; Emlen and Nijhout 1999, 2001; Moczek

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and Emlen 1999, 2000; Simmons et al. 1999; Palestrini et al. 2000).

Onthophagus taurus and *O. illyricus* are the only two European species in the subgenus *Onthophagus* s.str. (Zunino 1979), and are considered as sister species due to their striking morphological (Balthasar 1963; Baraud 1992; Krell and Fery 1992; Moczek and Emlen 1999; Martín-Piera and López-Colón 2000) and genetic similarity (Pizzo et al. 2006b).

Onthophagus taurus shows a typical Turanic-European-Mediterranean distribution (Balthasar 1963). Between 1975 and 1984 it was introduced into Australia and Tasmania as part of a biocontrol programme (Tyndale-Biscoe 1990), and into the United States due to one accidental and subsequent intentional releases. The chorology of *O. illyricus* is Turanic-European; its distribution widely overlaps with that of *O. taurus*. However, its exact range is still imprecisely known due to the unreliability of some distributional data (Martín-Piera and López-Colón 2000). In their extensive overlap zone, the two species sometimes occur in syntopy (Pizzo et al. 2006b; unpublished collecting results by the authors). Moreover, besides sharing the same pedotrophic (paracoprid) behaviour (Halffter and Matthews 1966; Moczek 1998), the two species even seem to share the same ecology and microhabitat: in the sympatric populations sampled by the authors, their nests can be found under the same dung pads, and differences in food selection were never detected (Pizzo et al. 2006b; unpublished data). The phenology, however, is slightly different: *O. taurus* has two population peaks during spring and summer, while *O. illyricus* peaks in the summer only. Moreover, *O. taurus* is able to colonise habitats at slightly higher altitude than *O. illyricus* (Martín-Piera and López-Colón 2000).

According to traditional classification criteria (Janssens 1960; Paulian and Baraud 1982), differences between *O. taurus* and *O. illyricus* mainly concern the surface of the elytra. *Onthophagus illyricus* elytra show granulation on the sutural intervals (interstriae) and are pubescent almost throughout, whereas those of *O. taurus* exhibit uneven intervals with pubescence only in the apicodiscal part. Punctures of the pronotal declivity of *O. taurus* are weaker than those on the disc, whereas this difference is not appreciable in *O. illyricus* (Krell and Fery 1992). All other external characters can be considered as identical in the two species. The shapes of structural components of the genitalia provide another fairly reliable diagnostic character for the identification of these species (Zunino 1971; Paulian and Baraud 1982), but in some cases a certain degree of interspecific overlap in the shapes of copulative structures remains (Pizzo et al. 2006a, 2008).

In this paper we test the usefulness of the shape of the adult epipharynx, an internal foraging trait, as a tool to reveal differentiation patterns between *Onthophagus taurus* and *O. illyricus*. The epipharynx (Fig. 1) is a lobe-like structure on the inner surface of the clypeus; it is largely membraneous but also contains a large, fairly complicated sclerite (which is studied here), and it is rich in sensilla. In Coleoptera the epipharynx was studied first in larval stages, and then its adult structure was also used in systematics and in phylogenetic analyses (Dellacasa 1983; Barbero et al. 2003; Medina et al. 2003; Philips et al. 2004). In Scarabaeoidea the epipharynx was studied comparatively by Nel and Scholtz (1990). However, its shape has never been analysed quantitatively.

The interspecific discriminatory power of a morphological structure can be biased by conditions increasing its range of intraspecific variation. For example, a trait could be highly dimorphic between sexes or strongly dependent on ecophenotypic variation, making interspecific comparisons difficult. Therefore, we first investigated patterns of within-species variation in epipharynx shape to test the effects of the possible

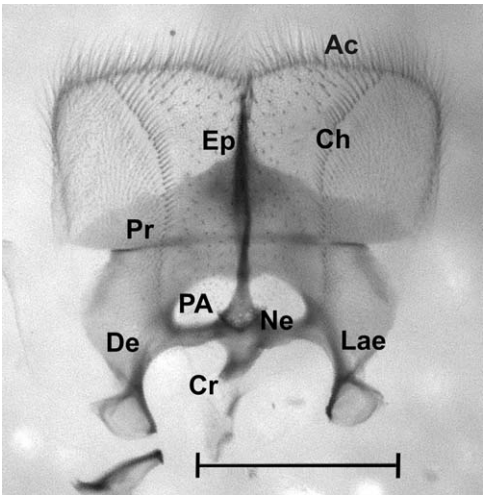

Fig. 1. *Onthophagus taurus*, adult epipharynx, distal margin at top. Scale bar = 0.5 mm. The structure is formed by a double, more or less thick membranous plate and by variously sclerotised parts (the tormae). It is situated on the inner surface of the clypeus, and is divided into a proximal and a distal part by the proplegmatium (Pr), a well-developed transversal plica (Nel and Scholtz 1990). Its distal margin is deeply notched in the middle, and lined with dense and long setae, the acropariae (Ac). The chaetopariae (Ch), a pair of longitudinal rows of thick setae, are well-developed, extending as far as the plegmatic area (PA). The latter is constituted by a pair of well-developed, non-sclerotised, oval membranes on the epipharynx surface. The anterior epitorma (Ep) is a narrow rod-like but strong, sclerotised structure. The nesium (Ne), is an arched, almost triangular subregion, with few evident sensilla. The crepis (Cr) is an asymmetrical unpaired apodeme. Dexiotorma (i.e. right torma; De) and laeotorma (i.e. left torma; Lae) carry the pternotormae, a pair of internally widened, symmetrical and rounded apodemes. For futher details refer to Dellacasa and Dellacasa (2006).

sources of intraspecific variation. Specific factors taken into account were sexual dimorphism, male horn polyphenism and covariation with head morphology, because the epipharynx is set in a hollow of the head and polyphenic horn development is correlated with variations in head morphology (Pizzo et al. 2006a; Macagno et al. 2009). Thereafter we tested interspecific discriminatory power of epipharynx shape.

We used a combination of linear measurements and a landmark-based geometric morphometrics approach (Bookstein 1991; Rohlf and Marcus 1993; Adams et al. 2004) to quantify inter- and intraspecific epipharynx shape differentiation between *O. taurus* and *O. illyricus*. We did this study for a single exemplary study site, at which the two species co-occur and where populations have been studied intensively in preceding years (Pizzo et al. 2006a, 2006b, 2008). While this surely limits the general validity of our results, it was useful to keep sample sizes manageable.

Static allometries and trade-offs: conceptual framework

As in previous studies (e.g. Emlen 2001), we used static allometries as the first step to point out correlations between morphological traits. Allometry refers to disproportionately large or small changes in one body part relative to the size of the body as a whole, which usually implies disproportionate investment, or resource allocation, during development (Shingleton et al. 2007). The development of any trait involves potential cost in terms of resources. Thus, on the premise that resources for growth are limited during ontogenesis, most traits should be involved in trade-offs with other morphological or functional features (Minelli 2004, 2007). Therefore, the allometry of each trait is likely to be affected by the allometry of every other trait (Bonduriansky and Day 2003). Nijhout and Wheeler (1996) remarked on the unique condition under which adult structures of holometabolous insects grow. The metamorphosing individual does not feed during the pupal stage. Therefore, imaginal structures grow within a virtually closed system in which body parts are in direct competition for metabolic resources (Roth and Mercer 2000). Organ competition was experimentally demonstrated first in flies (Smith and French 1991) and butterflies (Nijhout and Emlen 1998). In dung beetles of the genus *Onthophagus*, the production of horns reduces the size of neighbouring body parts such as antennae, eyes or wings, depending on the cephalic or thoracic location of the horns (Nijhout and Emlen 1998; Emlen 2001).

The respective precursors of adult horns and mouthparts develop from imaginal discs that proliferate during the same prepupal growth stage (Svacha 1992; Moczek et al. 2007). Thus it is likely that their development is mediated, at least in part, by the same developmental mechanisms (Truman and Riddiford 2002). These considerations, together with the spatial proximity between horns and epipharynx, suggested a possible competition for resources. We therefore looked for clues of developmental trade-offs between these body parts.

Moreover, since the process of evolutionary divergence in holometabolous insects often goes along with shifts in allometric slopes of functional traits (Weber 1990; Moczek and Nijhout 2003), epipharynx allometries were also used to find clues of interspecific differentiation.

Material and methods

Sampling

The geometric morphometric analysis was based on a sample of 149 *O. taurus* (53 females; 96 males, i.e. 54 'major' plus 42 'minor' ones) and 60 *O. illyricus* (20 females; 40 males = 20 'major' + 20 'minor') collected at La Mandria Natural Park (Venaria Reale, Turin, NW Italy). These two populations had been described in detail before, from both morphological and molecular points of view (Pizzo et al. 2006b). Specimens from that site can be assigned to either species unambiguously based on elytra and pronotum punctation and genital shape (Pizzo et al. 2006b).

Relative warp (RW) analysis of epipharynx shape variables and Partial Least Squares (PLS) analysis, employed to describe covariation between epipharynx and head shape, were performed on the whole sample. The epipharynx static allometry vs. pronotum size analyses were conducted on a subsample of 147 *O. taurus* (51 females; 96 males = 54 'major' + 42 'minor') and 57 *O. illyricus* (20 females; 37 males = 20 'major' + 17 'minor') from which both pronotum and epipharynx were available. Regressions between horn length and epipharynx shape variables, and the existence of a trade-off between horn length and epipharynx size were tested on 48 'major' and 37 'minor' males of *O. taurus*, and on 19 'major' and 16 'minor' males of *O. illyricus*.

Morphological measurements and statistical analyses

Morphological terminology

Certain parts of the scarabaeoid epipharynx are often referred to with different terms by various authors (Nel and De Villiers 1988). Here, we employ the terminology proposed by Barbero et al. (2003) (Fig. 1).

Morphological preparations

Individuals were cleaned in boiling distilled water for 10 min and dissected. Epipharynges were extracted and boiled for 5 min in 5% caustic potash. After 5 min in distilled water, they were dehydrated for 5 min in 70% and 5 min in 99% ethanol, then cleared in BioClear (CIAB, Chemical Instruments AB, Lidingö, Sweden) for 5 min. Epipharynges were mounted in Canada Balsam between microscope slides and coverslips. The Balsam volume was standardised for all preparations. Heads and pronota were then fixed separately on plasticine supports. Epipharynx, head and pronotum images were taken using a Leica Z16Apo stereoscopic dissecting scope (Leica Microsystems AG, Wetzlar, Germany) at magnifications of 57.5x (epipharynx), 32x (head), and 12.5x (pronotum).

Landmarks and measurements

In landmark-based morphometric analyses, the morphology of an object is represented by coordinates of sets of landmark points (Bookstein 1991). Landmarks, chosen for their ease of identification, homology

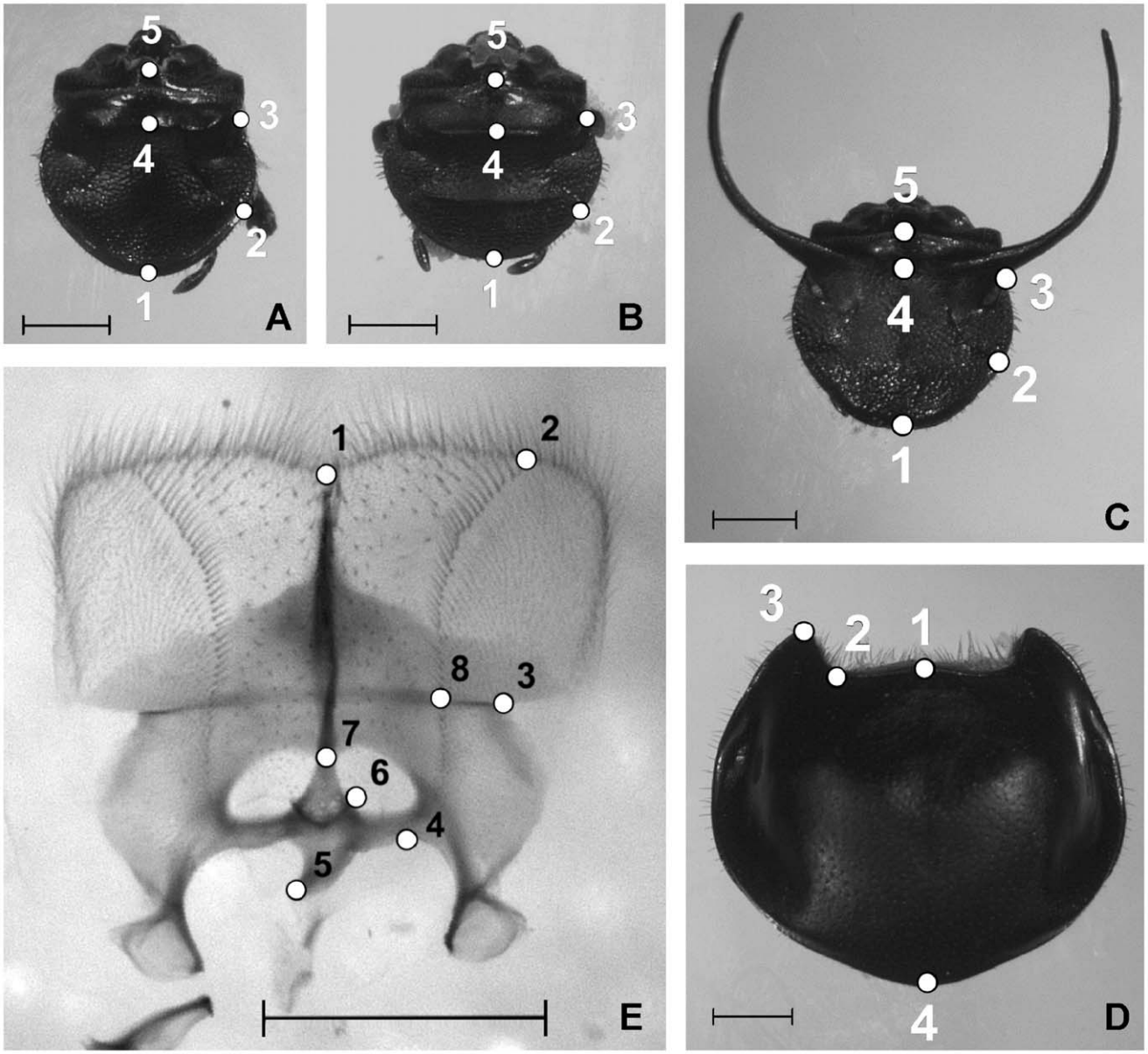

Fig. 2. *Onthophagus taurus*, positions of morphometric landmarks. (A) Head of 'minor' male. (B) Head of female. (C) Head of 'major' male. (D) Pronotum, dorsal side. (E) Epipharynx, ventral side. Landmarks (for definitions see text) were digitised on the left half of each structure to avoid effects of bilateral asymmetry. Scale bars A–D = 1.0 mm, E = 0.5 mm.

between the two species, and ability to capture the general shape of each morphological structure, were digitised using TpsDig 2.05 (Rohlf 2006a). To evaluate the confidence of the landmark configuration, a repeatability test was conducted as in Pizzo et al. (2008). Landmarks on the head (N = 5), pronotum (N = 4), and epipharynx (N = 8) were digitised as shown in Fig. 2. Landmarks of each specimen were optimally aligned using a Generalised Procrustes Analysis (GPA) to remove the non-shape effects of translation, rotation, and scale (Rohlf 1990, 1999; Rohlf and Slice 1990). As long as variation in shape space is small, the data in tangent space are an almost perfect approximation of the data in shape space; we tested this approximation with TpsSmall 1.20 (Rohlf 2003). We used the thin-plate spline (TPS) approach (Bookstein 1989, 1991; Rohlf 1999) to generate multivariate descriptions of the shape of each specimen, and to visualise them on deformation grids.

The landmarks were defined as follows: Head, left side: 1) clypeal margin, on midline; 2) genal suture at fore margin; 3) hind corner of eye; 4) frontal suture, on midline; 5) upper margin of occipital foramen, on midline. Pronotum, left side: 1) fore margin, at midline; 2) fore margin, at base of fore angle; 3) apex of fore angle; 4) hind margin, on midline. Epipharynx: 1) fore margin, at anterior epitorma; 2) chaetopariae, at fore margin; 3) side margin of proplegmatium; 4) lower junction point between laeotorma and the small transversal torma that links laeotorma and crepis; 5) apex of crepis; 6) left margin of nesium sensilla; 7) widening point of fore epitorma, at intersection with unsclerotised portion of plegmatic area; 8) chaetopariae, at joining point to proplegmatium.

Relative warp analysis of epipharynx shape

GPA, multivariate descriptions of the shape variables, relative warp analysis (the principal component analysis of the partial warp scores) and visualisation of transformation grids were performed using TpsRelw 1.45 (Rohlf 2007b) to describe intra- and interspecific epipharynx shape variations. The two first relative warp scores were plotted on an axis system using SPSS 14.0 software (SPSS Inc., Chicago, IL); parallel inspection of relative warp plots and transformation grids gave revealed trends in shape variation of the structure. Discriminant analysis was carried out on relative warp scores to obtain a classification matrix based on epipharynx shape variation. We used the percentages of correct classifications to evaluate whether *O. taurus* and *O. illyricus* specimens form two distinct groups.

Partial least squares analysis between epipharynx and head shapes

As pointed out previously (Pizzo et al. 2008), the PLS method is applied widely in many disparate research fields. In spite of its unquestionable usefulness (Streissguth et al. 1993; Abdi 2003), PLS analysis remains relatively little used in geometric morphometrics (McIntosh et al. 1996; Rohlf and Corti 2000; Roggero 2004; Zelditch et al. 2004; Bastir and Rosas 2006; Marugán-Lobón and Buscalioni 2006; Gomes and Monteiro 2008). Here, TpsPLS 1.18 (Rohlf 2006b) was employed to describe covariation between epipharynx and head shape. The hypothesised pattern of covariation of the two anatomical structures was tested through the cross-set analysis (r value representing the correlation degree) (Rohlf 2006b). The PLS method requires homogeneous samples, so we analysed *O. taurus* and *O. illyricus* separately.

Regression between horn length and epipharynx shape variables

To evaluate the possible influence of horn growth on epipharynx shape we performed a regression test between horn length and epipharynx shape variables using TpsRegr 1.34 (Rohlf 2007a). Horn length was measured following the outer edge of the horn as in Moczek (2006), using the software LAS v 2.5.0 (Leica Application Suite).

Trade-off between horns and epipharynx

Onthophagus taurus and *O. illyricus* horn lengths were graphed as a function of body size (Eberhard and Gutierrez 1991; Emlen and Nijhout 2000; Moczek and Nijhout 2003), and their scaling relationships were determined by fitting to the data the four-parameter non-linear regression

$$y = y_0 + \frac{ax^b}{c^b + x^b}$$

(Moczek et al. 2004), where x is body size, y is horn length, y_0 specifies the minimum horn length, a defines the horn length range in the sample, b represents a slope coefficient, and c is body size at the point of inflection of the sigmoid curve. Epipharynx vs. pronotum centroid size scaling relationships were assessed with a linear regression model ($y = y_0 + bx$). Developmental trade-offs between horns and epipharynx were looked for as negative correlations between residuals of each scaling relation (Emlen 2001). Best fitting equations, calculation of residuals and their correlation were performed with the software package SigmaPlot (Systat Software Inc., Richmond, CA).

Epipharynx static allometry

In geometric morphometrics, the value of centroid size (the square root of the sum of squared distances of the set of landmarks from their centroid, measured on calibrated images) can be used as a proxy for the size of a structure (Alibert et al. 2001; Rosenberg 2001; Ubukata 2003; Pizzo et al. 2006a). We used the centroid size values as an estimation of epipharynx size, and the pronotum centroid size as an inference on general body size. These log-transformed variables were regressed with SPSS 14.0. We tested the homogeneity of the slopes of regression lines found for *O. taurus* and *O. illyricus*, respectively, by including the interaction term between the covariate (log-transformed pronotum centroid size) and the factor (species) in an analysis of covariance using Statistica 6.0 (StatSoft Inc., Tulsa, OK) (Engqvist 2005).

Results

The TpsSmall analysis (correlation coefficient $r = 1.000$, slope $= 0.9996$, root of mean squared error $= 0.000025$) confirmed very good correspondence between shape space and tangent space, thus indicating that the geometrical heterogeneity of the sample was small enough to allow subsequent analyses.

Sexual dimorphism

In the relative warp (RW) analysis of epipharynx shape, the respective first RW score explains 17.32% of the total variation in *O. taurus*, 18.89% in *O. illyricus*; the second score explains 15.51% in *O. taurus*, 15.85% in *O. illyricus* (Fig. 3). The plots of these two first RW scores display consistent overlap between the sexes, hence clearly show that the epipharynx is a non-dimorphic structure.

Male polyphenism

In the RW analysis of male epipharynx shape, the respective first RW score explains 21.12% of the total variation in *O. taurus*, 22.54% in *O. illyricus*; the second score explains 15.32% in *O. taurus*, 18.32% in *O. illyricus*. Male morphs largely overlap in the plot of the two first RW scores (not figured), suggesting that epipharynx shape does not vary as a function of male polyphenism.

Covariation between epipharynx and head shape

In the PLS analysis between epipharynx and head shapes, six dimensions were computed for each species. Values of r (i.e. the coefficients of correlation between shape vectors 1 and 2) for each dimension were low and similar in the two species. Most of the covariation is concentrated in the respective first two dimensions, but although all permutation tests gave significant p-values (<0.01), covariation values were essentially negligible (<0.001) (Table 1).

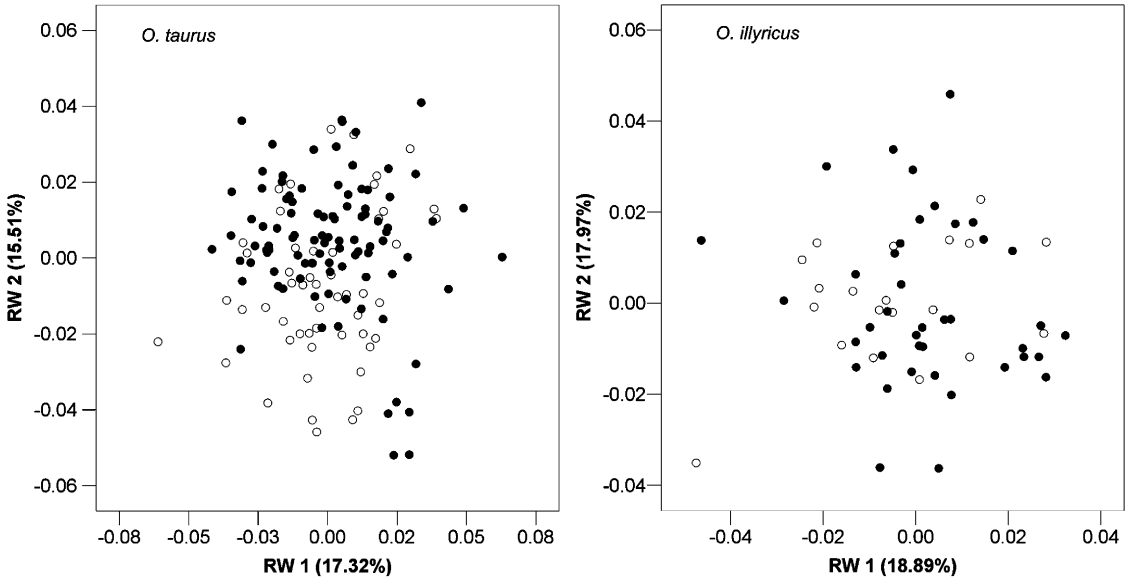

Fig. 3. Scatterplots of the respective first two relative warp (RW) scores obtained from the separate analyses of epipharynx shape in *Onthophagus taurus* and *O. illyricus*; percentage of shape variation explained by each RW reported in brackets. Open circles = females; solid circles = males. Epipharynx shape does not permit intraspecific discrimination of the sexes.

Table 1. Results of Partial Least Squares analysis of shape vectors 1 (head) and 2 (epipharynx) for *Onthophagus taurus* and *O. illyricus*.

Species	Dimension	Covariance	Correlation coefficient *r*	Explained covariation (%)
O. taurus	1	$2.33 \cdot 10^{-4}$	0.53	56.72
	2	$1.86 \cdot 10^{-4}$	0.49	35.93
	3	$7.15 \cdot 10^{-5}$	0.29	5.32
	4	$3.29 \cdot 10^{-5}$	0.28	1.13
	5	$2.38 \cdot 10^{-5}$	0.11	0.59
	6	$1.71 \cdot 10^{-5}$	0.12	0.30
O. illyricus	1	$1.72 \cdot 10^{-4}$	0.54	43.94
	2	$1.55 \cdot 10^{-4}$	0.42	35.85
	3	$9.91 \cdot 10^{-5}$	0.39	14.61
	4	$4.85 \cdot 10^{-5}$	0.28	3.50
	5	$3.18 \cdot 10^{-5}$	0.30	1.50
	6	$2.00 \cdot 10^{-5}$	0.32	0.60

Regression between horn length and epipharynx shape

Regression between horn length and epipharynx shape as described by partial warps gave significant results both for *O. taurus* (Wilks' Lambda = 0.63, $F_{12,72} = 2.762$, $p < 0.001$; Generalised Goodall *F*-test: $F_{12,996} = 2.96$, $p < 0.001$; Permutation test = 0.20%) and *O. illyricus* (Wilks' Lambda = 0.39, $F_{12,21} = 2.76$, $p = 0.02$; Generalised Goodall *F*-test: $F_{12,384} = 2.96$, $p = 0.019$; Permutation test = 2.60%). However, the deformation predicting epipharynx shape as a function of horn length (provided by deformation grids; not figured) was very low and barely detectable.

Trade-off

Onthophagus taurus and *O. illyricus* horn length/body size scaling relationships were respectively represented by the two four-parameter non-linear regressions

$$y = 0.2 + \frac{3.6x^{26.2}}{3.37^{26.2} + x^{26.2}} (R^2 = 0.91)$$

and

$$y = 3.86 + \frac{54.32x^{3.65}}{0.34^{3.65} + x^{3.65}} (R^2 = 0.78).$$

Epipharynx and pronotum centroid size scaling relationships were assessed with the two linear regression models $y = 0.21 + 0.20x$ for *O. taurus* ($R^2 = 0.99$) and $y = 0.08 + 0.22x$ for *O. illyricus* ($R^2 = 0.90$); correlation of residuals of the first and the second regression did not reveal any evidence of negative trade-offs in either species ($y = 0.01x$, $R^2 = 0.05$ for *O. taurus*; $y = 0.005x$, $R^2 = 0.32$ for *O. illyricus*).

Interspecific shape differences

In the RW analysis aimed at describing epipharynx shape variation between *O. taurus* and *O. illyricus*, including all specimens regardless of sex and morph, the first RW score explains 22.71% of the total variation, the second 14.04%: only minimal overlap between specimens of the two species is detectable (Fig. 4). Discriminant analysis performed on RW scores showed that 100% of *O. illyricus* and 98% of *O. taurus* were classified correctly (Wilks' Lambda = 0.23, $\chi^2 = 299.31$, $p < 0.001$).

Visualisation of the deformation grids made it possible to describe trends of epipharynx shape variation between the two species: visual inspection of these warp grids showed that generally the epipharynx of *O. taurus* is more compressed laterally (Fig. 4: landmarks 2, 3 and 8). In *O. illyricus*, on the other hand, the part of the plegmatic area between nesium (landmark 6) and proximal edge of anterior epitorma (landmark 7) is more compressed and narrower. This appears to be the most variable area in both species, while the anterior epitorma, the laeotorma and the crepis (landmark 5) are slightly less variable. Additionally, the crepis seems shorter in *O. taurus*, with the apex more arched; the chaetopariae show a small amount of interspecific dimorphism, and they are more rectilinear in *O. taurus*, more arched and stouter in *O. illyricus* (landmarks 2 and 8).

Epipharynx static allometry

Regressions between the log-transformed values for pronotum and epipharynx centroid size were significant both for *O. taurus* ($y = -0.472 + 0.803x$; $R^2 = 0.74$; $F_{1,145} = 413.467$; $p < 0.001$) and *O. illyricus* ($y = -0.587 + 0.974x$; $R^2 = 0.812$; $F_{1,55} = 237.913$; $p < 0.001$). The statistical comparison between the two regression lines showed that epipharynx scaling is different between the two species: the two regression lines differ significantly in their angular coefficient (species*log-transformed pronotum centroid size effect: $F_{1,200} = 6.16$; $p = 0.013$). Regression plots are shown in Fig. 5.

Discussion

The goal of this study was to evaluate the potential of the adult epipharynx as a tool for revealing differentiation patterns between closely related *Onthophagus* species. A combination of traditional and geometric morphometric techniques, which provide a powerful tool for highlighting subtle, yet significant, morphological modifications (Rohlf and Marcus 1993; Baylac et al. 2003), were chosen to gain insight into epipharynx

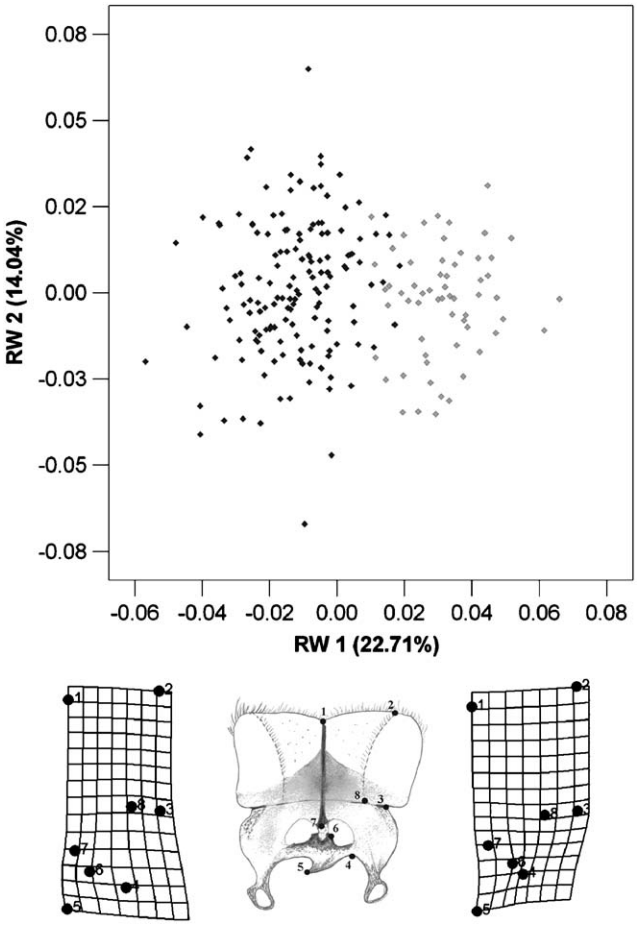

Fig. 4. Epipharynx shape differentiation between *Onthophagus taurus* and *O. illyricus*. Top: scatterplot of first two relative warp (RW) scores obtained from epipharynx analysis; black diamonds = *O. taurus*, grey diamonds = *O. illyricus*. Bottom left and right: TPS transformation grids for specimens plotted on the left (*O. taurus*) and right (*O. illyricus*) extremity of first RW axis. Bottom center: consensus shape.

differentiation patterns. A morphological character is reliable for interspecific discrimination if it is characterised by low intraspecific variability relative to interspecific variation. Therefore, analyses were conducted by first assessing the effects of all possible sources of intraspecific variation (sexual dimorphism, male horn polyphenism and covariation with head morphology) on epipharynx shape. Secondly, the reliability of interspecific variation patterns was analysed quantitatively.

Intraspecific variation

In both *Onthophagus* species, epipharynx shape is indistinguishable between females and 'major' and 'minor' males, as evident from the relative-warp plots. In males, the regression between horn length and epipharynx shape, although slightly significant, does not support any consistent modifications of the structure in response to horn development (deformation grids depict negligible shape variation).

Since the epipharynx is placed in a cavity of the head (anterior pharynx wall), one might have expected variation in its shape reflecting that of the head. Sex- and morph-dependent head shape variation has been highlighted in some *Onthophagus* species (Pizzo et al. 2006a; Macagno et al. 2009), where the head undergoes a morphological readjustment to accommodate horns of considerable bulk and disproportionate length. In *O. taurus* and *O. illyricus*, female heads differ only slightly from those of 'minor' males. The latter have longer, narrower heads, which can be beneficial –compared to those of 'major' males – when running through narrow tunnels to reach a female (Moczek and Emlen 2000; Pizzo et al. 2006a). In 'major' males the head is larger, more rounded and compressed longitudinally, thus giving the appearance of a massive build that could be advantageous in fighting (Pizzo et al. 2006a). Nevertheless, the analysis of covariation between epipharynx and head shape did not reveal a shared trend of variation.

The absence of epipharynx shape variation between the sexes and between the male morphs, as well as the absence of covariation with general head shape, is expected on the basis of strong constraints related to the feeding habits of individuals. The epipharynx plays an extremely specific role in that it is used to filter and select food particles. Considering the strict association of *Onthophagus* species with particular feeding resources (Halffter and Matthews 1966), stabilising selective pressures are likely to act on this structure, so that optimal functionality is maintained independent of sex and morph (Palestrini et al. 2000).

The close proximity between epipharynx and horns and their common and synchronous developmental patterns suggest a potential developmental trade-off between epipharynx and horns (Emlen 2001). Under this hypothesis, a negative correlation between residuals of epipharynx/pronotum and horn length/pronotum size scaling relationships could have been expected. On the contrary, our results showed that, despite the mentioned predisposing conditions, epipharynx size is not influenced by costs associated with horn development. Nijhout and Emlen (1998) demonstrated that in *Onthophagus taurus* the allocation of resources to horn development results in reductions in relative size of the compound eyes. The fact that the epipharynx does not show trade-off effects of horn development might suggest that the feeding function is more important than the visual one for the ecology of the species (Halffter and Matthews 1966). Under this scenario, epipharynx size and shape appear to be strongly

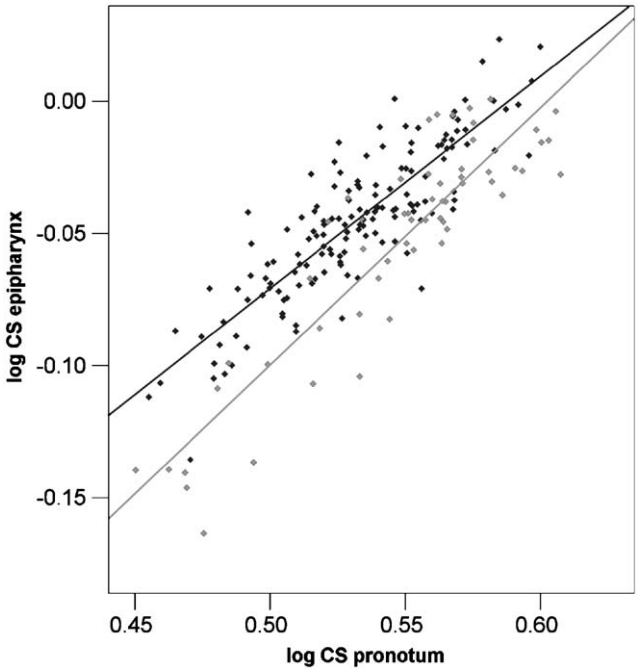

Fig. 5. Scaling relations between log-transformed centroid size (CS) values for pronotum and epipharynx in *Onthophagus taurus* (black diamonds) and *O. illyricus* (grey diamonds).

canalised. In general, trade-offs are strongest when the traits develop simultaneously, and when the developmental processes involved are similar (Nijhout 1994). *Onthophagus* horns and epipharynx satisfy both of these conditions; nevertheless our results suggest that these features may have independent and compartmentalised developmental trajectories. Developmentally compartmentalised traits tend to incur highly canalised resource use, and this may facilitate independent evolution of the traits.

Interspecific variation

In a study conducted on 36 species of the genus *Phalops* (Scarabaeinae), Palestrini et al. (1995) showed that, within each species, epipharynx general morphology and chaetotaxis (the number and spatial arrangement of setae on the ventral epipharynx surface) are quite constant. On the basis of qualitative inspection of the structure, no sexual dimorphism was detected in any species. However, different, independent evolutionary forces seem to be able to model this structure. It is likely that general shape and chaetotaxis reflect different evolutionary mechanisms. The general shape of the structure might depend on phylogenetic constraints, because it varies between species as a function of their phylogenetic relationships. Chaetotaxis, in contrast, should be more subject to adaptive convergence, because it is very similar among groups that are phylogenetically remote but live in similar environments (Barbero et al. 2003). This hypothesis is supported by the results of Verdù and Galante (2004), who showed that one of the most evident adaptations to xeric conditions is a shared trend of modification of the mouthparts such as the epipharynx, especially in those characters connected with chaetotaxis. Species adapted to the use of dry dung (e.g. *Onthophagus emarginatus*, *O. punctatus*, *O. latigena*) exhibit strongly developed setae on the epizygum, zygum, and apices of the acropariae.

Onthophagus taurus and *O. illyricus* are both soft-diet consumers. Where their distribution ranges overlap the two species occur not only sympatrically but also syntopically, and can be found to feed on exactly the same resources and on the same dung pads. Accordingly, their chaetotaxies do not show any of the described environmental adaptations; on the contrary, setae are thin and disposed in a corresponding pattern in the two species. However, when shape attributes of the epipharynx are considered, small but nonetheless significant differences clearly arise. Our results show that even if the first relative warp score only explains about 23% of the total variation (Fig. 4), the modifications that it describes are sufficient to discriminate the species, as demonstrated by the extremely high percentages of correct classifications obtained from discriminant analysis. Such differences have been quantitatively assessed here for the first time and, on the basis of previous considerations, there is scant evidence that they might have arisen as a consequence of different ecological adaptations (e.g. dry vs. soft resource use). Moreover, molecular data have provided evidence of a historical genetic isolation that persists today, whereas there is no evidence for any historical or recent ecological differentiation between *Onthophagus taurus* and *O. illyricus* (Pizzo et al. 2006b). Therefore, interspecific differences in the epipharynx might be the outcome of some secondary effects of allopatric speciation, e.g. of stochastic events (genetic drift or mutations in genes indirectly involved in defining epipharynx shape) that occurred at early stages of the divergence process. Selective constraints on more functional variants may have canalised and maintained the differentiation.

In this species pair, a clear genetic divergence corresponds to a moderate genitalic differentiation and a very subtle morphological divergence in external traits (Pizzo et al. 2006a, 2006b). Surprisingly, the epipharynx makes a better character for distinguishing the two species than the genitalia, at least when using geometric morphometric tools. This could be partially due to the fact that landmark positioning is easier on the rather flat epipharynx than on a three-dimensional structure (aedeagus, vagina).

In both species, the allometric coefficients found are as expected of body traits that are not under sexual selection, i.e. equal to or slightly lower than 1.0 (Harvey and Pagel 1991). The only published work on the *O. taurus* epipharynx (Palestrini et al. 2000) reported epipharynx allometric values lower than those of external body traits and only slightly higher than those of genitalia. In that paper, the authors used traditional morphometrics to obtain four dimensional variables on the epipharynx (height of epitorma, width of half the apical portion of epipharynx, width of half the plegmatical area, height of plegmatical area). They interpreted their results to suggest that this internal trait undergoes developmental processes and is subjected to selective pressures quite different from those affecting external traits. Palestrini et al. also indicated the possibility that a developmental trade-off has acted on this trait. In contrast, we found an almost isometric relationship. A possible explanation of these differences is that traditional measures might underestimate actual dimensional variation. Centroid size, instead, is able to take into account the overall dimensional variation, and may represent better the actual allometric relationships between traits.

Another aspect that provides evidence for this interspecific differentiation pattern is the fact that *O. taurus* and *O. illyricus* differ in their scaling relations (or static allometries; Shingleton et al. 2007) between epipharynx and pronotum centroid sizes. In *Drosophila melanogaster* it has been demonstrated that much additive genetic variance exists for individual dimension of wings, such that wing allometry can be changed in any direction by drift (Weber 1990). If a similar pattern applies to the epipharynx in *Onthophagus*, the divergence in epipharynx static allometry between the two sister species may be interpreted as an effect of allopatric speciation. This divergence is even more significant when considering that the two species occupy the same trophic niche. This may be additional circumstantial proof that the main process that drove epipharynx interspecific differences was stochastic and not caused by selection towards specific adaptations.

Further analyses of epipharynx variation over the entire geographic range of these and other sister species could offer a more complete characterisation of the trait and its usefulness for taxonomic purposes.

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